

## ADP-ribosylation of the $M_r$ 83,000 stress-inducible and glucose-regulated protein in avian and mammalian cells: Modulation by heat shock and glucose starvation

(*in vivo* ADP-ribosyl acceptor/heat shock-inducible proteins/two-dimensional gel electrophoresis)

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**ABSTRACT** ADP-ribosylation of proteins was analyzed by *in vivo* labeling of cells with [ $^3\text{H}$ ]adenosine, followed by separation of their protein components by two-dimensional isoelectric focusing/NaDodSO<sub>4</sub> polyacrylamide gel electrophoresis. We show here that in several cell types of avian and mammalian origin the major [ $^3\text{H}$ ]adenosine acceptor *in vivo* is a polypeptide with a  $M_r$  of 83,000 and isoelectric point of  $\approx 5.3$ . This polypeptide is identical to one of the stress-inducible and glucose-regulated proteins (here called SP83) previously described in avian and mammalian cells. Snake venom phosphodiesterase digestion of purified  $^3\text{H}$ -labeled SP83 releases 5'-AMP and a minor fraction of 2'-(5"-phosphoribosyl)-5'-AMP. *In vitro* labeling with [ $^{32}\text{P}$ ]NAD<sup>+</sup> of total cell lysates made in the presence of non-ionic detergents also results in incorporation of radioactivity into SP83. Both of these results strongly suggest that the modification is an ADP-ribosylation. Heat shock and glucose starvation of cells induce a rapid and extensive decrease in the incorporation of ADP-ribose into SP83, suggesting that ADP-ribosylation may be important for the regulation of the function of this protein.

ADP-ribosylation occurs as a posttranslational modification of a small group of proteins. The reaction is usually thought to be catalyzed and enhanced by toxins such as cholera or diphtheria toxin, and, in the cases examined, a specific enzymatic or regulatory function is known to be inhibited by the covalent attachment of ADP-ribose to the protein acceptors (1–5). Recently, several structural proteins have been shown to be modified by ADP-ribosylation as well as a response to cholera or diphtheria toxins *in vitro* (6, 7). Most of these modifications have been characterized and shown to occur only *in vitro* by utilizing NAD<sup>+</sup> as an ADP-ribose donor. Which proteins function as cytoplasmic ADP-ribose acceptors *in vivo* is presently unknown. Another form of the same type of modification is the addition of a polymer of ADP-ribose to histones (8). This reaction occurs *in vivo* (9) as well as *in vitro* (8). Large T-antigen found in simian virus 40-infected and transformed cells is another example of a nuclear protein whose function might be modulated by poly-ADP-ribosylation *in vivo* (10).

There is a small group of polypeptides whose synthesis and accumulation is stimulated severalfold when cells are challenged with a variety of stress situations (11–16). The major stress-induced polypeptides in invertebrate as well as vertebrate cells have apparent  $M_s$  of 110,000, 100,000, 90,000, 83,000, 68,000–70,000, 27,000, 26,000, 24,000, and 22,000 (12, 15, 17). (They will hereafter be referred to as SP110, SP100, SP90, SP83, etc.) Comparing the molecular weights, isoelectric points, and partial peptide maps of the individual stress-induced proteins has

clearly established a high degree of homology between all cell types thus far investigated (12, 15–20). A certain variation is observed in the extent of induction and accumulation of these polypeptides in different organisms and cell types. However, the SP90, SP83, and SP68–70 seem to be universally induced (12, 15, 17, 19). Most, if not all, of the stress-induced polypeptides with  $M_s$  between 70,000 and 110,000 are also present in normal cells (13, 20), suggesting that they participate in important cellular regulatory functions.

Three polypeptides whose transcription and translation are increased in response to glucose starvation have been described in mammalian and avian cells (21–24). Their  $M_s$  are estimated to be 94,000–99,000, 78,000, and 54,000–58,000, respectively (21, 23). Comparing the molecular weights and isoelectric points of these proteins (5.1, 5.3, and 5.9, respectively) with the stress-induced polypeptides, it is apparent that the  $M_s$  94,000–99,000 and 78,000 glucose-regulated polypeptides are similar to SP100 and SP83, respectively (13, 17, 21, 23). The identity of the  $M_r$  78,000 glucose-regulated protein with SP83 has recently been confirmed by peptide mapping (25).

We show here that the major ADP-ribose acceptor *in vivo* in avian and murine cells is identical to the  $M_r$  83,000 stress-induced and glucose-regulated protein. The ADP-ribosylation of this polypeptide is diminished by either heat shock or glucose starvation of avian and murine cells, respectively, suggesting that this chemical modification may play a pivotal role in the regulation of the function of SP83.

### MATERIALS AND METHODS

**Cell Cultures.** Primary chicken embryo fibroblasts (CEF) were prepared from 10- to 11-day-old embryos according to Rein and Rubin (26) and were grown in complete growth medium as described (27). The cultures were used between the third and seventh subcultivation. Stock cultures of the rat embryo cell line F2408 (called rat-1 cells) and rat-1 cell lines transformed with either SRD–Rous sarcoma virus (RSV) or the temperature-sensitive mutant LA24D of RSV were grown in Dulbecco's modified Eagle's medium (GIBCO) and 5% calf serum according to ref. 28. The normal and transformed rat cell lines were a kind gift of Richard Hynes (Massachusetts Institute of Technology).

***In Vivo* Labeling of Cells with [ $^3\text{H}$ ]Adenosine.** CEF or the rat cell lines were used at a density of  $2\text{--}4 \times 10^6$  cells per 100-mm-diameter tissue culture dish. Three to 4 hr prior to labeling, cells were fed with fresh medium. At the time of labeling

Abbreviations: iso-ADP-ribose, 2'-(5"-phosphoribosyl)-5'-AMP; RSV, Rous sarcoma virus; CEF, chicken embryo fibroblasts; araC, 1- $\beta$ -D-arabinofuranosylcytosine; CBB, Coomassie brilliant blue; SVP, snake venom phosphodiesterase; EF-2, elongation factor 2.

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cells were transferred to Dulbecco's modified Eagle's medium (with or without glucose, as described in figure legends) supplemented with 5% horse serum and were labeled for 6 hr with 75–125  $\mu\text{Ci}$  of [2,8,5'- $^3\text{H}$ ]adenosine (50.5 Ci/mmol; 1 Ci =  $3.7 \times 10^{10}$  Bq; New England Nuclear) per ml. CEF were labeled either in the absence or presence of 20  $\mu\text{g}$  of actinomycin D (Sigma) per ml and 10  $\mu\text{M}$  1- $\beta$ -D-arabinofuranosylcytosine (araC; Sigma). Cycloheximide was added to a final concentration of 100  $\mu\text{g}/\text{ml}$ . Inhibitors were added to the cell cultures 30 min prior to addition of the isotope. As indicated, 8-bromo-adenosine 3',5'-cyclic monophosphate (0.5 mM; Sigma) was added during the last 60 min of incubation with the isotope. Similarly, cells exposed to elevated temperature were incubated at 45°C during the last 60 min of labeling.

After the labeling period cells were washed twice with phosphate-buffered saline (pH 7.4), removed from the plate with a rubber policeman, and harvested by centrifugation ( $1,000 \times g$ , 3 min). The cell pellets were immediately dissolved in lysis buffer A (0.2% NaDodSO<sub>4</sub>/10 mM sodium phosphate, pH 6.9/2 mM EGTA/10 mM sodium fluoride/15% glycerol/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/60  $\mu\text{M}$  leupeptin) and were placed in a boiling water bath for 5 min. Solid urea was then added to give a final concentration of  $\approx 9$  M. The samples were subsequently analyzed by one- and two-dimensional gel electrophoresis (29). After staining gels with Coomassie brilliant blue-R (CBB) and destaining, the  $^3\text{H}$ -labeled gels were equilibrated in EN<sup>3</sup>HANCE (New England Nuclear) before drying, whereas  $^{32}\text{P}$ -labeled gels were dried directly. The dried gels were then exposed to Kodak X-Omat R (XAR-5) film at  $-70^\circ\text{C}$  for 2–21 days. Quantitation of fluorograms was done by scanning autoradiograms of one-dimensional gels in the linear range of exposure with a Quick Scan (Helena Laboratories, Beaumont, TX) gel scanner at 570 nm.

**In Vitro Labeling of Cell Lysates.** CEF were grown and harvested as described above. The resulting cell pellet was resuspended in 100  $\mu\text{l}$  of lysis buffer A containing 0.25% Nonidet P-40 instead of NaDodSO<sub>4</sub>. This lysis buffer was also supplemented with either 5  $\mu\text{Ci}$  of [adenylate- $^{32}\text{P}$ ]NAD<sup>+</sup> (800 Ci/mmol; New England Nuclear) per 100  $\mu\text{l}$  or 5  $\mu\text{Ci}$  of [ $\alpha$ - $^{32}\text{P}$ ]ATP (460 Ci/mmol; New England Nuclear) per 100  $\mu\text{l}$ . The lysates were incubated for 5 min at 37°C and the reaction was terminated by the addition of NaDodSO<sub>4</sub> to 0.2% and boiling the samples for 5 min. The samples were prepared for isoelectric focusing as above.

**Enzyme Digestion and TLC.** CEF were labeled with [ $^3\text{H}$ ]adenosine *in vivo* as described and proteins were separated by two-dimensional gel electrophoresis. From the CBB-stained and destained gels the area corresponding to SP83 (three isoelectric variants) was excised and the gel pieces were equilibrated for 2 hr in three 1-ml aliquots of 25 mM ammonium bicarbonate buffer (pH 8.0). Finally, the acrylamide pieces were resuspended in 400  $\mu\text{l}$  of ammonium bicarbonate and incubated at 37°C, and 20, 40, and 40  $\mu\text{l}$  of TPCCK-trypsin (10 mg/ml in ammonium bicarbonate; Worthington) were added, respectively, at intervals over a period of 48 hr. After this time, 1 mg of soybean trypsin inhibitor in a volume of 100  $\mu\text{l}$  was added to the sample and the incubation was continued for another 2.5 hr. By this treatment 83% of the total radioactivity present in the SP83 spot was released (see Table 1). To the supernatant obtained after trypsin digestion, MgCl<sub>2</sub> was added to 5 mM and snake venom phosphodiesterase [(SVP) from *Crotalus adamanteus*, EC 3.1.4.1, Worthington] was added to 100  $\mu\text{g}/\text{ml}$  ( $=4.4$  units/ml). The sample was then incubated for 2.5 hr at 37°C. To a 250- $\mu\text{l}$  aliquot of this sample, 50  $\mu\text{l}$  of 50% trichloroacetic acid was added, the mixture was kept on ice for 30 min, and precipitated protein was removed by centrifugation. The super-

natant was freeze-dried and redissolved in 10  $\mu\text{l}$  of distilled H<sub>2</sub>O. The SVP digest was spotted (0.5–2  $\mu\text{l}$ ) on a polyethylenimine-cellulose plate (Polygram R, CEL 300 PEI, Sybron, Brinkman), together with solutions of ATP, ADP, and AMP as references and was developed in 0.75 M Tris-HCl (pH 8.0) according to ref. 30. Reference nucleotides were detected under UV light. Radioactivity was analyzed by cutting the plate in 1-cm strips, eluting each piece with 750  $\mu\text{l}$  of 0.5 M HCl for 60 min, and dissolving the eluted samples in 20 ml of Aquasol (New England Nuclear) before liquid scintillation spectroscopy.

## RESULTS

**Incorporation of [ $^3\text{H}$ ]Adenosine into Proteins *in Vivo*.** In an attempt to analyze which protein(s) can function as ADP-ribose acceptors *in vivo*, we incubated different cell types with [ $^3\text{H}$ ]adenosine and analyzed total cellular proteins by two-dimensional isoelectric focusing/NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis. Once inside the cell, most of the labeled adenosine is rapidly incorporated into nucleic acid precursors and then into high molecular weight RNA and DNA. However, a part of the labeled intracellular [ $^3\text{H}$ ]ATP is used for the synthesis of [ $^3\text{H}$ ]NAD<sup>+</sup>, which is the immediate substrate for ADP-ribosylation of proteins. Because the majority of the  $^3\text{H}$  radioactivity is incorporated into nucleic acids, experiments were performed both in the absence and presence of nucleic acid synthesis inhibitors (actinomycin D and araC).

The results of these experiments are illustrated in Fig. 1. CEF (Fig. 1 A and B) and rat-1 fibroblasts (Fig. 1 C and D) were labeled for 6 hr with [ $^3\text{H}$ ]adenosine and total cellular proteins were separated by two-dimensional gel electrophoresis. As can be seen from the fluorograms (Fig. 1 B and D) there was only one major radioactive spot corresponding to a CBB-stained polypeptide. This polypeptide has an apparent  $M_r$  of 89,000

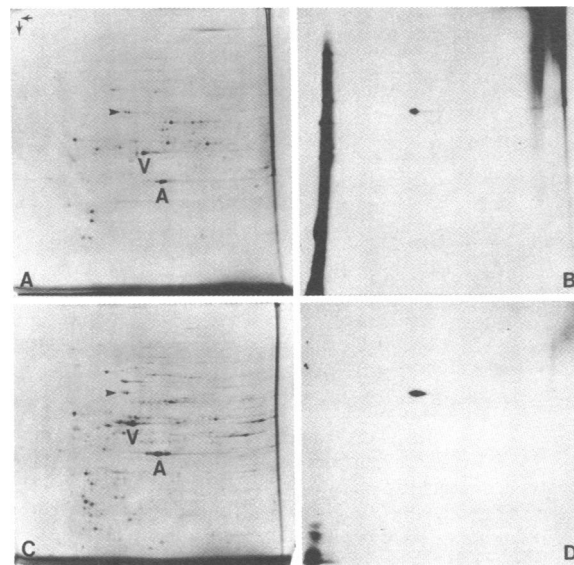


FIG. 1. Incorporation of [ $^3\text{H}$ ]adenosine into cellular proteins *in vivo*. CEF (A and B) and rat-1 cells (C and D) were labeled with [ $^3\text{H}$ ]adenosine *in vivo*. CEF were labeled in the absence and rat-1 cells in the presence of actinomycin D and araC. Total cellular proteins were then separated by two-dimensional isoelectric focusing/NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis. Proteins were visualized by CBB staining (A and C) and radioactively labeled material, by fluorography (B and D, respectively). The acidic end of the isoelectric focusing gel is to the left of the figures. Arrowhead points to the position of SP83. V, vimentin; A, actin. Internal  $M_r$  markers were: actin,  $M_r$  42,000; chicken vimentin,  $M_r$  52,000; SP70,  $M_r$  70,000;  $\alpha$ -actinin,  $M_r$  95,000; myosin heavy chain,  $M_r$  220,000; and fibronectin,  $M_r$  250,000 (see also Fig. 2).

and consists of at least three closely spaced isoelectric variants with pIs between 5.2 and 5.3. The most basic form of this protein did not incorporate [ $^3\text{H}$ ]adenosine, whereas at least two more acidic forms could be distinguished which incorporated radioactivity. The presence and absence of nucleic acid synthesis inhibitors gave identical results. However, in the absence of these inhibitors, radioactive material was detected both at the very acidic and basic ends of the isoelectric focusing gel (Fig. 1B; also see below).

The polypeptide labeled with [ $^3\text{H}$ ]adenosine had an identical  $M_r$  and isoelectric point to the  $M_r$  78,000–83,000 stress-induced and glucose-regulated protein previously described in avian and mammalian cells when run on identical gel systems (refs. 13, 17, 23, and 25; data not shown). The discrepancies in  $M_r$  determinations are most likely due to differences in molecular weight markers and gel electrophoresis systems used. Because this polypeptide has been previously assigned a  $M_r$  of 83,000, the [ $^3\text{H}$ ]adenosine-labeled polypeptide will be referred to hereafter as SP83.

When extracts identical to those described in Fig. 1 were analyzed by one-dimensional NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis, in principle, the same result was obtained (Fig. 2 A–F, lane 1). The major [ $^3\text{H}$ ]adenosine acceptor in CEF and rat fibroblasts was SP83. However, some minor labeled components were also observed with  $M_r$ s of about 100,000, 140,000, and 150,000. Under the conditions of labeling used here, histones H1 and H2b do not efficiently incorporate [ $^3\text{H}$ ]adenosine into poly-ADP-ribose and thus are not apparent in these gels (see *Discussion*). The SP83 polypeptide could also be labeled with [ $^3\text{H}$ ]adenosine in 6- to 8-day-old chicken embryo myotubes cultured *in vitro* (data not shown) and RSV-transformed rat-1 cells (Fig. 2 E and F, lanes 3 and 4). However, mature chicken erythrocytes did not incorporate any detectable  $^3\text{H}$  label into SP83 under similar labeling conditions (data not shown).

**The [ $^3\text{H}$ ]Adenosine Labeling of SP83 Occurs Posttranslationally.** Cells were labeled with [ $^3\text{H}$ ]adenosine in the presence of cycloheximide. As can be seen in Fig. 2 A–D (lane 3), the labeling of SP83 was not inhibited by cycloheximide at a con-

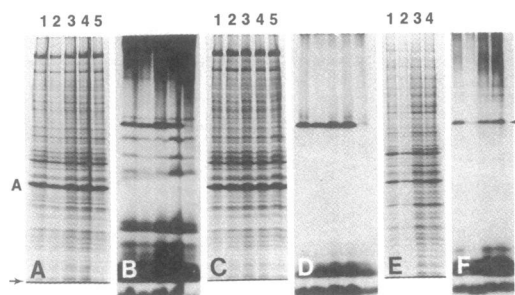


Fig. 2. Effect of various treatments of CEF and rat-1 cell lines on [ $^3\text{H}$ ]adenosine incorporation into SP83. (A–D) CEF were labeled with [ $^3\text{H}$ ]adenosine without (A and B) and with (C and D) actinomycin D and araC, and proteins were separated by one-dimensional NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis. Samples corresponding to  $\approx 1.5 \times 10^6$  cells were applied to each lane. Lane 1, untreated controls; lane 2, glucose-starved cells; lane 3, cells treated with 100  $\mu\text{g}$  of cycloheximide per ml; lane 4, cells treated with 0.5 mM 8-bromo-adenosine 3',5'-cyclic monophosphate during the last 60 min of labeling; and lane 5, cells incubated at 45°C for the last 60 min of labeling. (E and F) Rat-1 cell lines were analyzed as above after labeling cells with [ $^3\text{H}$ ]adenosine in the presence of actinomycin D and araC. Samples corresponding to  $\approx 0.35 \times 10^6$  cells were applied to each lane. Lane 1, rat-1, untreated; lane 2, rat-1, glucose-starved cells; lane 3, temperature-sensitive RSV (LA24D)-transformed rat-1, untreated; and lane 4, RSV-transformed rat-1, untreated. A, C, and E shows the CBB staining pattern, whereas B, D, and F shows the corresponding fluorograms. A, actin. Arrowhead points to SP83. The arrow in the left corner points to the dye front.

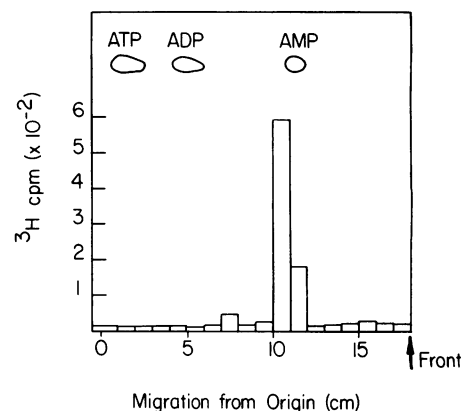


Fig. 3. TLC of SVP digestion products of *in vivo* [ $^3\text{H}$ ]adenosine-labeled SP83. SVP digest (1  $\mu\text{l}$ ) was spotted on a polyethylenimine-cellulose plate together with reference nucleotides and the chromatogram was developed and analyzed. Fractions 11 and 12 (5'-AMP) contain 79% of the radioactivity recovered from the plate, whereas fraction 8 contains 4.5%.

centration which is sufficient to inhibit >95% of protein synthesis in CEF (13). In fact, a slight increase (1.5 times the control) in the labeling of SP83 was observed when fluorograms were quantitated by scanning the films. However, this may be due to an increased specific activity of the  $\text{NAD}^+$  pool when the requirement of metabolic energy is decreased during the protein synthesis block. Furthermore, the incorporation of  $^3\text{H}$  into SP83 is as efficient in the presence of RNA and DNA synthesis inhibitors as in their absence (compare Fig. 2 B and D). Most RNA and DNA synthesis was inhibited under these conditions of labeling, except for some low molecular weight RNA species (see below).

These results conclusively show that the [ $^3\text{H}$ ]adenosine incorporation into SP83 is due to posttranslational modification of this protein. They further show that this modification is not linked to the synthesis of the protein, as has been shown to be the case for the methylation of SP83 and other heat shock-inducible proteins (31).

**SP83 is ADP-Ribosylated *In Vivo*.** To determine whether the [ $^3\text{H}$ ]adenosine incorporation into SP83 represents an ADP-ribosylation of the protein, two kinds of experiments were performed. Digestion with SVP of *in vivo* labeled and isolated SP83 and analysis of the digestion products by TLC were performed. As shown in Fig. 3 and Table 1, the main peak of radioactivity released from SP83 migrated at a position corresponding to 5'-AMP. This material represented 79% of the radioactivity recovered from the plate. Furthermore, a small peak of radioactivity (4.5% of total cpm recovered) consistently migrated at a position slightly in front of the ADP marker (fraction 8). This

Table 1. Quantitation of SVP digestion products released from *in vivo* [ $^3\text{H}$ ]adenosine-labeled and isolated SP83

Fraction	cpm	% recovery
Total	66,700	100
Solubilized with trypsin and SVP	55,200	83
TLC		
As 5'-AMP	36,200	54
As iso-ADP-ribose	2,050	3.1

CEF were labeled *in vivo* with [ $^3\text{H}$ ]adenosine and proteins were separated by two-dimensional gel electrophoresis. The SVP digestion of labeled and isolated SP83 and TLC of the digestion products was carried out as described in the legend to Fig. 3. Recovery was calculated on total cpm detected in SP83 isolated from five two-dimensional gels.

is a position expected for 2'-(5"-phosphoribosyl)-5'-AMP (iso-ADP-ribose), which would be released from a poly-ADP-ribose chain (10). These results suggest that SP83 is ADP-ribosylated.

To verify that SP83 is ADP-ribosylated (and not modified in any other way involving adenosine and 5'-AMP), we attempted to label the protein *in vitro*, with either [ $^{32}$ P]NAD $^{+}$  or [ $\alpha$ - $^{32}$ P]-ATP as substrates for the reaction. The result of the *in vitro* labeling with [ $^{32}$ P]NAD $^{+}$  is shown in Fig. 4. SP83 clearly incorporated  $^{32}$ P radioactivity from NAD $^{+}$ , further strengthening the suggestion that it is ADP-ribosylated. With [ $\alpha$ - $^{32}$ P]ATP as a substrate, no detectable radioactivity was incorporated into SP83 (data not shown).

Several other identifiable polypeptides can be efficiently labeled with [ $^{32}$ P]-NAD $^{+}$  *in vitro*. These include the acidic variants of vimentin and  $\beta$ -tubulin, two forms of tropomyosin, and the regulatory (G/F) subunit of the adenylate cyclase system (Fig. 4B). None of these polypeptides incorporated detectable amounts of [ $^3$ H]adenosine *in vivo*.

**Modulation of the ADP-Ribosylation of SP83 by Heat Shock and Glucose Starvation.** CEF were prelabeled for 5 hr with [ $^3$ H]adenosine at 37°C and then were incubated at 45°C for 60 min, still in the presence of the isotope. The level of ADP-ribosylation of SP83 decreased an average of 92% compared with untreated controls (Fig. 2B and D, lanes 1 and 5). Similar results were obtained with 8-day-old chicken embryo myotubes incubated at elevated temperature (data not shown).

Glucose starvation of rat-1 fibroblasts during the time of labeling also decreased the level of ADP-ribosylation of SP83. On the average one-fourth as much radioactivity was detected in SP83 as compared with cells grown under normal conditions (Fig. 2F, lanes 1 and 2). However, when chicken cells were incubated in the absence of glucose no change was detected in the incorporation of [ $^3$ H]adenosine into SP83 (Fig. 2B and D, lanes 1 and 2). This difference between avian and murine cells is most likely due to differences in metabolism and utilization of carbon sources. Furthermore, no significant difference in the  $^3$ H labeling of SP83 was observed in RSV-transformed rat-1 cells as compared with normal cells (Fig. 2F, lanes 1, 3, and 4). No alteration was observed in the incorporation of [ $^3$ H]-

adenosine into SP83 after treating CEF for 1 hr with 0.5 mM 8-bromoadenosine 3',5'-cyclic monophosphate (Fig. 2B and D, lane 4). However, when CEF and chicken embryo myotubes were labeled with  $^{32}$ PO $_4$  the incorporation of radioactivity into SP83 was enhanced after administering cAMP analogs to the cells (data not shown). This suggests that SP83 is both phosphorylated and ADP-ribosylated.

**Biosynthesis of Low Molecular Weight RNA: Effect of Stress Situations.** The [ $^3$ H]adenosine-labeled material other than SP83 that enters the NaDodSO $_4$ /polyacrylamide gels and migrates as discrete bands (see Fig. 2B) most likely represents discrete RNA species because: (i) on isoelectric focusing gels all of these bands migrate to the very acidic end of the gel (compare Figs. 1B and 2B, lane 1); (ii) these acidic species do not correspond to any CBB staining material (Fig. 1A and B); (iii) digestion with SVP of several of the acidic bands isolated from two-dimensional gels easily and in high yield releases [ $^3$ H]5'-AMP as the only radioactive nucleotide (data not shown); and (iv) most of their synthesis is inhibited by actinomycin D (compare Fig. 2B and D). Furthermore, several of the treatments of cells described in Fig. 2 appear to impair or increase the biosynthesis of certain specific low molecular weight RNAs. After glucose starvation or heat shock there is a substantial decrease in the labeling of some low molecular weight RNAs, whereas after a short treatment of cells with cAMP analogs an increase is observed in the labeling of several distinct but not all low molecular weight RNA species. Additionally, it appears that the synthesis or the copy number per cell of low molecular weight RNAs is increased in transformed cells as compared with normal cells. The physiological significance of these observations in relation to the biosynthesis and function of these low molecular weight RNAs and to the response to stress situations remains to be investigated.

## DISCUSSION

We have shown here that the major ADP-ribosyl acceptor in avian and murine cells *in vivo* is a polypeptide with a  $M_r$  of 89,000 and a pI of 5.3. This polypeptide is similar, if not identical, to the stress-induced and glucose-regulated protein (SP83) with a  $M_r$  of 83,000 previously described in avian and mammalian cells. The identity of the ADP-ribosylated polypeptide with SP83 can be made conclusively by comparing the position on two-dimensional gels of the [ $^3$ H]adenosine-labeled component and SP83 in relation to vimentin and SP70 (refs. 13, 17, 23, and 25; also *Results*). Digestion of a mixture of all isoelectric variants with SVP (see *Results*) and separation of the digestion products by TLC has shown that >80% of the modified molecules contain a single ADP-ribose moiety. This conclusion is also strengthened by the fact that *in vitro*, [ $^{32}$ P]NAD $^{+}$  is a much better substrate than [ $\alpha$ - $^{32}$ P]ATP for labeling of SP83.

SP83 has been reported to be both phosphorylated (ref. 17; unpublished data) and methylated (13, 31) *in vivo*. Furthermore, the methylation of SP83 has been shown to be coupled to the synthesis of the protein (31). On the other hand, ADP-ribosylation of the protein does not appear to be coupled to its synthesis and can occur posttranslationally.

Because it may be relevant to the function of the protein *in vivo*, it is of special interest that ADP-ribosylation of SP83 can be modulated by heat shock and glucose starvation. The response to heat shock is rapid and extensive and should not be due to changes in the specific activity of the NAD $^{+}$  pool, because cells were prelabeled for 5 hr before increasing the temperature. Furthermore, the removal of the ADP-ribose as a response to heat shock and glucose starvation takes place even under conditions in which the response to the stress situation

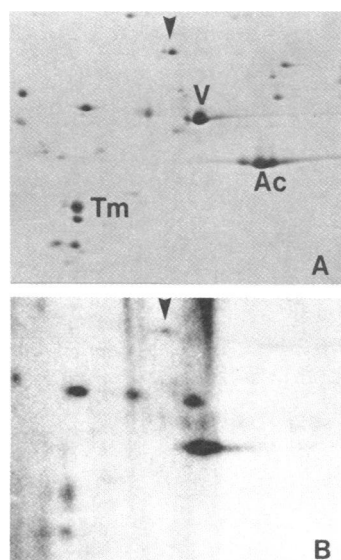


FIG. 4. *In vitro* labeling of CEF lysates with [ $^{32}$ P]NAD $^{+}$ . Lysates of CEF were prepared and labeled with [ $^{32}$ P]NAD $^{+}$  and proteins were separated by two-dimensional gel electrophoresis. (A) CBB-stained proteins; (B) the corresponding autoradiogram. The acidic end of the isoelectric focusing gel is to the left. Ac, actin; V, vimentin; and Tm, tropomyosin. Arrowhead points to the position of SP83.

(i.e., induction of the synthesis of SP83) is inhibited by the presence of RNA synthesis inhibitors. This implies that the loss of ADP-ribose from the protein is either independent of or precedes the induction of the protein. However, until the kinetics of labeling and disappearance of the modified group during stress situations have been investigated in greater detail, it is difficult to speculate more on these points.

Enzymes catalyzing the transfer of mono- or poly-ADP-ribose to protein acceptors have been described and characterized from several specific cytoplasmic locations (32–35). It is usually thought that poly-ADP-ribosylation is confined mainly to the nucleus, whereas cytoplasmic acceptors contain only mono-derivatives of ADP-ribose. Direct quantitation of ADP-ribose shows that there is  $\approx 0.1$ – $0.2\%$  poly-ADP-ribose of total ADP-ribose (36). Therefore, cytoplasmic acceptor(s) should be by far the most abundant species detected in *in vivo* labeling experiments. This is consistent with our findings that SP83 is the major ADP-ribose acceptor *in vivo* and explains why nuclear proteins like histones are not detected as being modified in these experiments.

In the experiments presented here we noted that the molecular weight and pI of the ADP-ribosylated SP83 is fairly similar to that of the protein synthesis elongation factor 2 (EF-2) (37). Together with the fact that SP83 is the major ADP-ribose acceptor *in vivo* and EF-2 is the major ADP-ribosylated protein *in vitro* in the presence of diphtheria toxin, this question warranted a direct comparison. Therefore, CEF lysates (prepared as in Fig. 4) were incubated with [ $^{32}$ P]NAD $^{+}$  in the absence and presence of purified diphtheria toxin subunit A (a kind gift of R. J. Collier, University of California, Los Angeles). The result shows that the major ADP-ribose acceptor in the presence of diphtheria toxin *in vitro* is not identical to SP83. The EF-2, as identified in this manner, has a  $M_r$  of 10,000–20,000 more than SP83 and an  $\approx 2$  pH unit higher pI.

There are two generalizations that can be made from the known properties of ADP-ribosylated proteins thus far studied in some detail: several mono-ADP-ribosylated proteins possess either a GTPase activity or a GTP binding site, whereas poly-ADP-ribosylated proteins are nucleic acid binding proteins. The first class of proteins includes EF-2, G/F regulatory subunit of adenylate cyclase, and tubulin, whereas the second class is exemplified by histones and the simian virus 40 T-antigen. Whether SP83 possesses a GTPase activity or nucleic acid binding site whose activity or binding properties can be modified by the addition of ADP-ribose to the protein remains to be investigated.

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